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Cannabinoid receptor type 1 antagonism significantly modulates basal and loud noise induced neural and HPA axis responses in male Sprague Dawley rats

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Abstract

Altered regulation of the hypothalamic-pituitary-adrenal (HPA) axis is associated with stress-induced changes in cognitive, emotional, and physical health. Recent evidence indicates that the endogenous cannabinoid (eCB) system may modulate HPA axis function both directly and more centrally, via regulation of limbic brain systems that control HPA axis activity. The current study examines the contribution of cannabinoid type 1 (CB1) receptor modulation throughout the neuraxis on control and stress-induced HPA axis activity. Adult male Sprague Dawley rats were given intraperitoneal injections of either CB1 receptor antagonist (AM251, 2mg/kg) or vehicle 30 minutes prior to a session of loud white noise stress (95 dBA for 30 min) or placement in a familiar sound-proof chamber. Immediately following stress and control treatments, rats were killed, the brains and pituitary glands were excised for subsequent immediate early gene (*c-fos* mRNA) measurement, and trunk blood was collected for subsequent determination of corticosterone (CORT) and adrenocorticotrophic (ACTH) hormone levels. AM251 treatment resulted in a potentiated plasma ACTH response to loud noise stress. AM251 treatment also increased stress-induced plasma CORT levels, but that increase may be due to an increase in basal plasma CORT levels, as was evident in control rats. AM251 treatment produced three distinctive *c-fos* mRNA response patterns across the various brain regions examined. In cortical (prelimbic, infralimbic, somatosensory, and auditory) and some subcortical structures (basolateral amygdala and paraventricular nucleus of the hypothalamus), AM251 treatment produced a substantial increase in *c-fos* mRNA that was comparable to the elevated *c-fos* mRNA levels present in those brain regions of both vehicle and AM251-treated stressed rats. In some other subcortical structures (bed nucleus of the stria terminalis and medial preoptic area) and the anterior pituitary, AM251 treatment produced a *c-fos* mRNA response pattern that was similar to the response pattern of ACTH hormone levels, i.e. no effect on no noise control levels, but an augmentation of stress-induced levels. Conversely, in the medial geniculate and ventral posterior thalamus, AM251 treatment inhibited stress-induced *c-fos* mRNA induction. These data indicate that disruption of eCB signaling through CB1 receptors results in potentiated neural and endocrine responses to loud noise stress, but also substantial increases in activity in various brain regions and the adrenal gland.

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Keywords

endocannabinoid; HPA axis; AM251; stress; noise

1. Introduction

Accumulating evidence implicates the endogenous cannabinoid (eCB) system as an important regulator of emotionality, and stress reactivity (Finn, 2010; Gorzalka and Hill, 2009; Hill et al., 2010; Lutz, 2009; Riebe and Wotjak, 2011; Valverde, 2005). eCB modulate central nervous system activity through two established ligands, anandamide (Devane et al., 1992) and 2-arachidonyl glycerol (2AG) (Sugiura et al., 1995), which are rapidly synthesized by specific enzymes in postsynaptic neurons in response to calcium-dependent synaptic signaling or other metabotropic receptor activation (Stella and Piomelli, 2001). Once produced, the highly lipid-soluble eCB interact with presynaptic eCB receptors and downstream second-messenger cascades, where they generally have been shown to inhibit glutamate, GABA, acetylcholine, norepinephrine, and serotonin release, among others (Freund et al., 2003; Schlicker and Kathmann, 2001). Both eCB ligands bind to type 1 cannabinoid receptors (CB1), which are the most widely expressed eCB receptors in the central nervous system (Herkenham et al., 1991), and to the centrally more restricted type 2 receptors (CB2) (Atwood and Mackie, 2010). In addition to their central nervous system actions, eCB also have well characterized peripheral actions through the same receptor subtypes (Atwood and Mackie, 2010). Given the widespread influence of eCB on neurotransmission, the overall contribution of eCB activity on specific neural functions has been difficult to precisely define.

Recent studies suggest that the endogenous cannabinoids negatively regulate stress responsiveness (Cota, 2008; Hill and McEwen 2010, Hill et al., 2009; Patel et al., 2004,2005; Tasker 2004). For instance, genetic deletion of CB1 receptors in mice results in hyperactive hypothalamo-pituitary-adrenal (HPA) axis responses to a variety of laboratory stressors, as indexed by increased plasma adrenocorticotrophic hormone (ACTH) and corticosterone (CORT) levels (Aso et al., 2008; Cota et al., 2007; Haller et al., 2004; Steiner et al., 2008; Uriguen et al., 2004). CB1 receptor knockout mice also express a heightened circadian peak of plasma CORT and impaired glucocorticoid negative feedback compared to wildtype mice (Cota et al., 2007). Furthermore, systemic pharmacological antagonism of CB1 receptors in mice potentiates CORT release in response to restraint and forced swim stress (Patel et al., 2004; Steiner et al., 2008) as well as stress-induced neuronal activity (as indexed by Fos) in the paraventricular nucleus of the hypothalamus (Patel et al., 2004), cingulate cortex, lateral septum, and nucleus accumbens (Patel et al., 2005). On the other hand, increasing the availability of anandamide by systemic pharmacologic blockade of the enzyme responsible for its degradation results in reduced CORT release to restraint stress (Patel et al., 2004, 2005).

Much of the reported work examining the role of eCB activity in stress and HPA axis regulation has been performed using mice, though rats have been used in examining the specific involvement of the basolateral amygdala (Hill et al., 2009) and hypothalamic nuclei (Di et al., 2003, 2005a,b, 2009; Evanson et al., 2010; Ginsberg et al., 2010). Increases in regional Fos protein expression in multiple limbic regions were observed in mice after administration of a CB1 receptor antagonist followed by restraint challenge, suggesting that the stress modulatory effects of eCB are not limited to the HPA axis (Patel et al., 2005). To our knowledge, no published work has examined the effects of acute stress and CB1 receptor antagonism on *c-fos* mRNA expression in rats. Differences in reported patterns of stressor-induced limbic eCB levels in mice (Patel et al., 2004, 2005; Rademacher et al.,

2008) compared to rats (Hill et al., 2007, 2009) suggest possible species differences that warrant further investigation of the functional contribution of eCB processes in stress responses in rats. In addition, no studies have explored the possibility that CB1 receptor antagonism modifies sensory processing that might then be reflected in limbic and hypothalamic structures, in response to stress (Patel et al., 2005). The current study was therefore undertaken to assess regional *c-fos* mRNA induction in several sensory, limbic, and hypothalamic nuclei, as well as pituitary (ACTH) and adrenal (CORT) hormone responses to CB1 receptor antagonism on control and acute loud noise exposure. We chose to examine *c-fos* mRNA in contrast to Fos protein because of the rapid induction and transient expression of mRNA after the onset of neuronal signaling. *c-fos* mRNA likely provides a tighter temporal representation than Fos protein of relative neural activity to proximal events immediately preceding brain harvesting.

Materials and Methods

2.1 Subjects

Forty-two male Sprague-Dawley rats (Harlan, Indianapolis IN) weighing 275–300 grams upon arrival were used. Animals were housed in polycarbonate tubs containing wood shavings, with wire lids providing rat chow and water ad libitum. Conditions in the animal colony were controlled to constant humidity and temperature, with a 12:12 hour light/dark cycle (lights on at 7:00 am). Testing was performed between 8:30 am and 12:30 pm during the circadian nadir for the HPA axis. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Colorado and conformed to the United States of America National Institute of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and the number of animals used.

2.2 Acclimation

Animals were allowed two weeks of acclimation to the colony before testing. The first week, animals were housed in groups of four. During the second week of acclimation, rats were individually housed and handled daily, in the colony room, from days one through four. On each of the last three days before testing, rats were transported in their home cages from the colony to the testing room, handled, returned to their home cages, and placed inside individual acoustic chambers (without noise exposure) for thirty minutes. This pre-exposure was intended to familiarize the rats to all of the testing procedures and minimize novelty related responses on the test day.

2.3 Drug Treatment

Rats were randomly assigned to one of four groups: Vehicle treated and noise exposed (n=10), Vehicle treated controls (n=10), AM251 treated and noise exposed (n=12), and AM251 treated controls (n=10), in a 2×2 balanced design. The CB1 antagonist/inverse agonist AM251 (Ascent Scientific, Princeton, NJ) was used to assess the involvement of the endogenous cannabinoid system on control and acute loud noise exposure. AM251 was dissolved in dimethyl sulfoxide (DMSO), Tween 80, and physiological (0.9%) saline (in a 1:1:8 ratio, respectively). We experienced difficulty in keeping AM251 from precipitating out of solution, so on the testing day, a stir plate was used to maintain suspension, and syringes were loaded immediately prior to dosing. AM-251 treated rats received a single intraperitoneal injection of 2 mg/kg, in injection volumes of 1 ml/kg. This dosage was chosen based on pilot testing in our laboratory suggesting that this dose was adequate to produce enhancement of loud-noise induced HPA axis activity. On the test day, rats received a single intraperitoneal injection of AM251 or a similar volume of vehicle (DMSO/Tween 80/0.9% saline) 30 minutes prior to placement in the acoustic chambers.

2.4 Loud Noise Procedures

The acoustic chambers used in this experiment have been described in detail in Day et al. 2009. On the testing day, rats were placed in the acoustic chambers in their home cages thirty minutes after vehicle or AM251 injection. Rats were either kept under quiet “no noise” control conditions (background noise of fans approximately 57 dB SPL - A scale) or loud noise (95 dB) was turned on immediately and remained on for thirty minutes. Immediately upon noise termination or quiet chamber exposure, rats were removed from the acoustic chambers, sacrificed by decapitation, and trunk blood was collected in chilled EDTA-containing Vacutainer tubes. Brains and pituitary glands were immediately harvested and frozen in -30 to -4° C isopentanes.

2.5 Corticosterone Enzyme Linked ImmunoSorbent Assays (ELISA)

The corticosterone assay was performed according to the manufacturer's instructions (AssayDesigns, Ann Arbor, MI) with one modification. Ten microliters of plasma in the standard buffer were placed in a hot water bath (65°C) for one hour instead of using the steroid displacement reagent. Levels were quantified on a BioTek Elx808 microplate reader and calculated against a standard curve generated concurrently.

2.6 ACTH Immunoradiometric Assay (IRMA)

Plasma (200 μl) was assayed for levels of ACTH using an Immunoradiometric Assay kit (Diasorin, Stillwater, MN, USA), according to the manufacturer's instructions. Briefly, the plasma was incubated overnight with a ^{125}I -labelled monoclonal antibody specific for ACTH 1–17, a goat polyclonal antibody specific for ACTH 26–39, and a polystyrene bead coated with a mouse anti-goat antibody. Only ACTH 1–39 in the sample bound both antibodies to form an antibody complex. Beads were washed to remove unbound radioactivity, counted with a gamma counter, and the concentrations of ACTH determined by comparison with a standard curve generated concurrently. All samples from this study were run in the same assay.

2.7 In situ Hybridization

The method for *in situ* hybridization histochemistry has been previously described (Day and Akil, 1996). Briefly, 12 μm sections were cut on a cryostat (Leica model 1850), thaw-mounted on polylysine-coated slides and stored at -80°C . A [^{35}S]-UTP-labeled riboprobe against *c-fos* mRNA (680 mer; courtesy of Dr. T. Curran, St Jude Children's Hospital, Memphis TN) was generated using standard transcription methods. Sections were fixed in 4% paraformaldehyde (1 hour), acetylated in 0.1 M triethanolamine with 0.25% acetic anhydride (10 min.) and dehydrated through graded alcohols. Sections were hybridized overnight at 55°C with a [^{35}S]-UTP-labeled riboprobe diluted in hybridization buffer containing 50% formamide, 10% dextran sulfate, $2\times$ saline sodium citrate (SSC), 50 mM PBS, pH 7.4, $1\times$ Denhardt's solution, and 0.1 mg/ml yeast tRNA. The following day, sections were treated with RNase A, 200 $\mu\text{g}/\text{ml}$ at 37°C (1 hour), and washed to a final stringency of $0.1\times$ SSC at 65°C (1 hour). Dehydrated sections were exposed to X-ray film (BioMax MR; Eastman Kodak, Rochester, NY) for structure-appropriate times (1–3 weeks) and the films analyzed as described below. Structures chosen for *c-fos* mRNA analysis include regions with high levels of neuronal activity (as indicated by induction of *c-fos* mRNA) in response to acute loud noise stress (Burow et al., 2005).

2.8 Semi-quantitative x-ray film analysis

Levels of *c-fos* mRNA were analyzed by computer-assisted optical densitometry. Anatomical landmarks were based on the white matter distribution of unstained tissue sections, according to a standard rat brain atlas (Paxinos and Watson, 1998). Brain sections

were captured digitally (CCD camera, model XC-77; Sony, Tokyo, Japan), and the relative optical density of the x-ray film was determined using Scion Image version 4.0 for PC. A macro was written (Dr. S. Campeau) that enabled signal above background to be determined automatically. For each section, a background sample was taken over an area of white matter, and a signal threshold was calculated as mean gray value of background + 3.5 standard deviation. The section was automatically density sliced at this value, so that only pixels with gray values above these criteria were included in the analysis.

2.9 Statistical analyses

SPSS version 18.0 was used to perform statistical analyses (Chicago, IL, USA). Values for plasma CORT and ACTH as well as *c-fos* mRNA expression were analyzed using a 2-way analysis of variance (ANOVA), with stress treatment (no noise vs. loud noise) and drug treatment (vehicle vs. AM251) as fixed factors. Significance for all tests was established at a $P = 0.05$. All data presented in the figures are listed as mean values $\pm$ standard error. Outlier values in the data set were identified as those being greater than 2 standard deviations from the mean when included in the data set, and were excluded.

3. Results

3.1 Endocrine responses

ACTH—Analysis of plasma ACTH revealed significant main effects of stress ($F_{1/34} = 6.59$, $p = 0.02$) and AM251 drug treatment ($F_{1/34} = 35.7$, $p < 0.01$), as well as a significant interaction between drug and stress ($F_{1/34} = 5.46$, $p = 0.03$; Fig. 1), indicating that CB1 receptor antagonism by AM251 treatment significantly potentiated the noise stress-induced increase in ACTH.

CORT—Analysis of plasma CORT from trunk blood taken immediately after sacrifice revealed significant main effects of loud noise stress exposure ($F_{1/37} = 217$, $p < 0.01$) and AM251 drug treatment ($F_{1/37} = 16.8$, $p < 0.01$), but not a significant interaction between stress and drug treatment ($F_{1/37} = .29$, $p = 0.6$; Fig. 1). These statistical results indicated that both loud noise exposure and AM251 treatment significantly increased levels of plasma CORT.

3.2 *c-fos* mRNA expression

3.2a HPA axis-intrinsic structures

Anterior Pituitary Gland: Analysis of *c-fos* mRNA expression in the anterior pituitary glands revealed that there were significant main effects of loud noise stress exposure ($F_{1/33} = 320$, $p < 0.01$) and AM251 drug treatment ($F_{1/33} = 7.8$, $p = 0.01$), as well as a significant interaction between loud noise exposure and antagonism of CB1 receptors with AM251 ($F_{1/33} = 6.4$, $p = 0.02$ Fig. 2). Taken together, these results indicated that systemic AM251 treatment resulted in a potentiation of *c-fos* mRNA expression, but only in response to loud noise stress exposure in anterior pituitary gland tissue. This pattern is similar to the potentiation of response measured in plasma ACTH by interaction of loud noise exposure and AM251.

Paraventricular Nucleus of the Hypothalamus: Expression of *c-fos* mRNA in the paraventricular hypothalamic nucleus (PVN) was found to be significantly increased by AM251- compared to vehicle-treated controls, but was not significantly potentiated by AM251 in the noise stress condition (Fig. 2). Significant main effects of loud noise stress ($F_{1/37} = 53.3$, $p < 0.01$) and AM251 treatment ($F_{1/37} = 18.9$, $p < 0.01$) indicated that both loud noise stress and AM251 treatment significantly increased *c-fos* mRNA expression in

the PVN. Analysis revealed a near-significant trend of interaction between loud noise stress exposure and AM251 treatment in *c-fos* mRNA expression ($F_{1/37} = 3.91$, $p = 0.06$). It should be noted that several film exposure times were tested to minimize a possible ceiling effect of the radionuclide exposure on the film, especially in the AM251/loud noise condition. In contrast to the other areas quantified using an appropriately shaped template, each PVN section were individually traced, reflecting the normal variance in shape and size of this region. We report the average signal of this structure rather than the integrated density, which is importantly influenced by the exact measurement area.

3.2b Stress-reactive sub-cortical brain structures—Expression of *c-fos* mRNA in additional forebrain and midbrain structures revealed significant increases in response to loud noise stress in all areas quantified as compared to vehicle-treated rats. Antagonism of CB1 receptors by systemic administration of AM251 resulted in three distinct patterns of *c-fos* mRNA induction: 1- Significant potentiation of stress-induced *c-fos* mRNA induction without reliable increases above control effects; 2- Significant potentiation of *c-fos* mRNA induction in the control condition without further increase following noise stress; and 3- Significant reduction in *c-fos* mRNA induction by AM251 treatment, especially in the noise stress condition.

Structures found to display significant potentiation of stress-induced *c-fos* mRNA induction by AM251 included the anteroventral bed nucleus of the stria terminalis (BNST) ($F_{1/38} = 5.69$, $p < 0.01$) and medial preoptic area of the hypothalamus (MPA) ($F_{1/37} = 6.31$, $p = 0.02$) as measured by significant interactions between loud noise exposure and AM251 drug treatment (Fig. 4). Though statistically insignificant, the interaction patterns of *c-fos* mRNA expression in the lateral septum ($p = 0.1$) were similar to that of the MPA and BNST, with no increase in *c-fos* mRNA resulting from AM251 treatment without noise, but potentiated induction when combined with loud noise stress treatment. The ventral lateral septum was quantified separately, and expressed a non-significant ($p > 0.05$) pattern similar to the lateral septum (data not shown). In contrast, *c-fos* mRNA expression in the basolateral amygdala (BLA) was substantially increased by AM251 drug treatment with and without loud noise (Fig. 4). Analysis of the BLA revealed significant main effects of loud noise stress exposure ($F_{1/37} = 5.32$, $p = 0.03$) and AM251 treatment ($F_{1/37} = 21.3$, $p < 0.01$), without significant interaction ($F_{1/37} = 0.69$, $p = 0.41$). Surprisingly, in both thalamic areas measured, antagonism of CB1 receptors by AM251 treatment resulted in a pattern of inhibition of *c-fos* mRNA expression in stressed rats (Fig. 5). This distinct interaction effect was measured in the somatosensory thalamus (VP: combined measure of ventral posterolateral (VPL) and ventral posteromedial (VPM) nuclei) ($F_{1/37} = 6.05$, $p = 0.02$), but a significant interaction was not measured in the auditory medial geniculate nucleus (MGN) ($F_{1/36} = 1.77$, $p = 0.19$, Fig. 5).

3.2c Cortical Areas—Significant main effects of loud noise stress were measured in the auditory ($p < 0.01$), prelimbic ($p < 0.01$), and infralimbic cortices ($p < 0.01$), but not in the somatosensory cortex ($p = 0.1$). AM251 treatment significantly increased *c-fos* mRNA induction in the auditory ($p < 0.01$), somatosensory ($p = 0.01$), and prelimbic cortices ($p = 0.04$), but not in the infralimbic cortex ($p = 0.23$). The effects of CB1 receptor antagonism on *c-fos* mRNA expression were not significantly potentiated by exposure to loud noise stress in any of these cortical regions (Fig. 6). Significant interactions between loud noise stress exposure and AM251 drug treatment were measured in the somatosensory cortex ($F_{1/38} = 4.8$, $p = 0.04$), prelimbic cortex ($F_{1/37} = 4.26$, $p = 0.05$), and infralimbic cortex ($F_{1/37} = 5.89$, $p = 0.02$), but not the auditory cortex ($F_{1/38} = 0.75$, $p = 0.39$). AM251 induced a very similar pattern of *c-fos* mRNA induction in all four of these cortical regions, which may approach a response ceiling in the stress condition.

4. Discussion

The results of this study contribute to the knowledge of the involvement of endocannabinoid signaling in the nervous system of male Sprague Dawley rats in several ways. Plasma corticosterone and ACTH were examined as indices of stressor-induced HPA axis activation, and *c-fos* mRNA expression was used as a measure of recent neural activity (Kovacs, 1998). Vehicle treated rats displayed increases in HPA axis activity, and widespread induction of *c-fos* mRNA in response to an acute thirty minute loud noise exposure, as previously reported (Burow et al., 2005, Campeau and Watson 1997). Antagonism of type 1 eCB receptors by intraperitoneal administration of 2 mg/kg AM251 altered either control and/or stress-induced responses for all measures. In most cases AM251 treatment increased the control or stress-induced levels of HPA axis activity and regional *c-fos* mRNA induction. These results are consistent with previous reports that disruption of inhibitory eCB signaling increases HPA axis activity in non-stressed and acute stress conditions (Aso et al., 2008; Cota et al., 2007; Haller et al., 2004; Hill and McEwen, 2010; Patel et al., 2004, 2005; Steiner et al., 2008; Uriguen et al., 2004). Interestingly, however, AM251 treatment inhibited stress-induced levels of *c-fos* mRNA in the measured thalamic nuclei. The data suggest that the CB1 receptor antagonist-mediated potentiation of HPA axis response to acute loud noise stress was related to modulation of limbic processing of the stressor. The increases in neural and endocrine activity from administration of AM251 in the no-noise condition may suggest a widespread involvement of eCB signaling in regulating activity in non- or low-stressed conditions. It is important to consider that measures taken from rats administered vehicle or AM251 without noise stress may reflect activity related to stress from the injection procedure, and may not reflect a true “non-stressed” condition. It would be of interest to habituate all rats to the stress of the i.p. injection procedure for several days before the testing day, or administer the drug and vehicle remotely via surgically implanted catheters to eventually answer this question.

Acute antagonism of CB1 receptors in control condition

HPA axis activation—Recent studies have suggested the possibility of tonic inhibitory eCB tone constraining HPA axis output through interactions at the level of the basolateral complex of the amygdala (Hill et al. 2009) and paraventricular hypothalamic nucleus (Di et al. 2009, Hill and McEwen 2010, Patel et al. 2004, 2005). One hour after administration of AM251, robust induction of *c-fos* mRNA in the basolateral complex of the amygdala and paraventricular hypothalamic nucleus were measured, and also corresponded to an increase in plasma corticosterone. However, this was observed without a significant concurrent elevation of circulating ACTH levels or *c-fos* mRNA induction in the anterior pituitary gland 1 hr after treatment, reducing the possibility that the elevation of basal corticosterone was due to a prolonged effect of AM251 treatment on anterior pituitary-mediated neurohormone secretion. It is possible, however, that there was a transient stimulatory effect of AM251 treatment on basal ACTH secretion which was missed by the 60 minute delay of the measurements, which would also explain the more sustained elevation of corticosterone. If such was the case, then it also raises the possibility that the increased CORT measured in the plasma from AM251-treated control rats reflected potentiation of a putative transient activation of the HPA axis by the mild stress of the i.p. injection procedure, rather than a true increase in adrenocortical basal activity (Galiegue et al., 1995). An alternative explanation for the increase in CORT measured in AM251-treated control rats is disruption of a tonic eCB-mediated inhibition of activity at the level of the adrenal cortex.

CB1 antagonist mediated increases in neural activity—Of interest, significant increases in *c-fos* mRNA were observed in control AM251-treated rats in all cortical areas examined, including the prelimbic, infralimbic, somatosensory, and auditory cortices. Due to

generally high CB1 receptor levels in all cortical areas (Herkenham et al., 1991), this observation may reflect generally increased cortical activity locally due to removal of tonic eCB-mediated cortical inhibition. Alternatively, higher cortical *c-fos* mRNA induction might be secondary to higher levels of sensory-dependent basal thalamic activity, but this possibility seems unlikely because there was no corresponding increase of *c-fos* mRNA in the thalamic medial geniculate and ventroposterior nuclei. Higher basal *c-fos* mRNA expression in the basolateral complex of the amygdala and the paraventricular hypothalamic nucleus after AM251 treatment, may also be due to either local disinhibition of eCB1 receptors, or increased inputs from cortical or other brain regions. Localized administration of AM251 into the basolateral amygdaloid complex or paraventricular hypothalamic nucleus will be necessary to further characterize these effects and to understand specific contribution of eCB signaling in these structures during both drug treatment and acute stress. Again, measures collected from control rats in this experiment may reflect activity related to the mild stress of the i.p. injection procedure, which in the case of AM251-treated control rats, would possibly reflect CB1 antagonist-mediated potentiation of this activity.

The lack of significant potentiation of *c-fos* expression measured in some areas (e.g. cortical areas, BLA, PVN) does not rule out local eCB involvement in acute stress responsiveness. Our measures are taken from a single time point immediately after a 30 minute exposure to loud noise stress. Recent work by Hill et al., (2011) has shown that disruption of eCB activity in the prefrontal cortex leads to a prolongation of HPA axis response to acute stress. It is possible that AM251 could affect the time course of activity in a pattern not detectable at the time of our measurements, which may have been visible if tissue was taken from brains at a later time after the acute stress session.

AM251-mediated potentiation of responses to stress

Antagonism of CB1 receptors with AM251 resulted in potentiation of the acute HPA axis response to a thirty-minute presentation of loud noise stress, as measured by plasma ACTH. The high levels of circulating plasma corticosterone obtained with loud noise exposure might have precluded a clear observation of AM251-induced potentiation of CORT levels. Analysis of *c-fos* mRNA induction in anterior pituitary gland tissue supported this pattern of potentiation. The increased response to loud noise did not appear to be explained by AM251-mediated potentiation of activity specifically at the level of the paraventricular nucleus of the hypothalamus, although a ceiling effect cannot be ruled out. This conclusion is consistent with the finding by Evanson et al. (2010) that microinfusion of AM251 into the paraventricular nucleus of the hypothalamus did not alter the HPA axis response to acute stress challenge. Significant AM251-induced potentiation of *c-fos* mRNA responses to loud noise stress was only observed in some of the limbic regions known to project to the paraventricular nucleus of the hypothalamus, including the anterior bed nucleus of the stria terminalis (anteroventral nuclei), the medial preoptic area, and a similar trend of induction was measured in the lateral septum. Surprisingly, none of the cortical structures measured displayed potentiated *c-fos* mRNA induction beyond the levels observed to loud noise exposed vehicle-treated rats, perhaps again reflecting a ceiling effect. Importantly, AM251 inhibited stress-induced induction of *c-fos* mRNA in the medial geniculate body, and the ventroposterior thalamic nuclei. This thalamic inhibition could be a result of the elevated cortical activity in primary sensory cortex that provides feedback on thalamic sensory relay nuclei (Briggs & Usrey, 2007). Regardless of whether the inhibitory effects of AM251 on thalamic neural activity is direct or indirect, a possible consequence is alteration of thalamic processing during loud noise exposure, and perhaps other stress situations, which is a novel finding with regard to eCB signaling and stress interactions. A possible disruption of sensory perception of the stressor may also explain the non-additive effects of AM251 and stress in the cortical regions investigated. The inhibition of activity in the sensory regions of

AM251-treated, noise-stressed rats is consistent with the findings of Ho et al., (2010) who reported localized subdural administration of a CB1 receptor antagonist to inhibit activity in the whisker barrel cortex in response to whisker movement. It should be noted that reduction of the medial geniculate body functions by neurochemical lesions or inactivation with muscimol reliably inhibits HPA axis responses to loud noise stress (Campeau et al., 1997, Day et al., 2009). The prediction, from these observations, would be that the AM251-mediated reduction in *c-fos* mRNA induction during loud noise exposure in the auditory thalamus would reduce HPA axis responses. At the very least, these results minimize the possibility that AM251 was potentiating HPA axis activity and *c-fos* mRNA induction in several limbic regions by simply potentiating activity in subcortical sensory processing regions, as AM251 induced a reduction in loud noise-induced *c-fos* mRNA levels in thalamic regions, including the medial geniculate body. Taken together, these data further suggest that CB1 antagonism specifically increases HPA axis response to loud noise stress by potentiating activity in limbic structures projecting to the HPA axis, perhaps together with potentiation of intrinsic HPA axis structures (i.e., anterior pituitary).

It is possible that the increased limbic response to loud noise stress in rats treated with AM251 would be associated with increased emotional and/or cognitive perception of the stimulus as threatening. Increased sensitivity to activation in neuronal systems responsible for cognitive perception or processing of a stimulus as threatening, or in neural structures involved in the subsequent heightening of arousal in response to perceived threat may result in a heightened response to the situation and may lead to disproportionately large psychological and/or physical reaction. Evidence to support a relationship of eCB deficiency to increased psychological response to stressful circumstance was reported in 2004 by Haller et al., who found that CB1 KO mice exhibited increased anxious behaviors in a context-dependent manner.

The increased neural and endocrine responses to loud noise stress measured in rats that were administered AM251 in this study suggest that deficiency in eCB signaling at CB1 receptors would likely lead to increased sensitivity to psychological stress. Increased responsiveness to repeated psychological stressors would likely result in exacerbation of physical and emotional changes resulting from chronic stress. In this way, deficiency in neural eCB signaling may be an initiating factor in the etiology of stress-induced disruption of health. eCB deficiency may also be involved in the perpetuation of physical and psychological state of increased vulnerability to stress given reports that both chronic restraint stress and chronic glucocorticoid administration result in widespread reductions in limbic eCB signaling (Hill et al. 2008, 2009b; Gorzalka et al. 2008). Taken together, this evidence suggests that deficiency in neural eCB signaling may be an important aspect in understanding the interrelationship of physical and psychological factors involved in a downward spiral of chronic stress-induced pathology.

- Antagonism of CB1 receptors potentiated neural and endocrine responses to loud noise stress.
- AM251 administration alone also increased activity in various brain regions adrenal gland.
- AM251 inhibited neural response to loud noise stress in the VP nucleus and MGN of the thalamus.

Abbreviations

ACTH	adrenocorticotrophic hormone
ANOVA	analysis of variance
BLA	basolateral amygdala
BNST	bed nucleus of the stria terminalis
CORT	corticosterone
dB	decibel
DC	drug-treated non-stressed control
DMSO	dimethyl sulfoxide
DN	drug-treated noise-stressed
eCB	endocannabinoid
HPA	hypothalamus-pituitary-adrenal
MPA	medial preoptic area of the hypothalamus
PVN	paraventricular hypothalamic nucleus
SSC	saline sodium citrate
VC	vehicle-treated non-stressed control
VN	vehicle-treated noise-stressed
VPL	ventral posterior lateral thalamus
VPM	ventral posterior medial thalamus

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Fig. 1. Plasma ACTH and corticosterone levels in response to drug and stress treatment. Top: AM251 treatment potentiated the noise stress-induced increase in plasma ACTH. Significant main effects of loud noise stress (#, $p < 0.02$); AM251 drug treatment (*, $p < 0.01$), and significant interaction of loud noise stress and AM251 treatment (†, $p < 0.03$). Bottom: AM251 treatment and loud noise stress increase circulating corticosterone levels. Significant main effects of loud noise stress (#, $p < 0.01$), and AM251 drug treatment (*, $p < 0.01$).

Fig. 2. *c-fos* mRNA induction in structures intrinsic to the HPA axis. A. AM251 treatment significantly increased loud noise stress-induced *c-fos* mRNA in the anterior pituitary gland ($\dagger$, $p < 0.02$). Significant main effects of loud noise stress ($\#$, $p < 0.001$) and AM251 drug treatment ($*$, $p = 0.01$). B. Representative autoradiograph of *c-fos* mRNA expression in the anterior pituitary gland of a rat in no-noise condition, with quantified region of interest marked by a circle. Scale bar = 1 mm. C. Representative pituitary gland autoradiograph from a rat exposed to loud noise stress. D. AM251 ($*$, $p < 0.01$) and loud noise stress ($\#$, $p < 0.01$) significantly induced *c-fos* mRNA expression in the paraventricular nucleus of the hypothalamus. E. Representative autoradiograph of *c-fos* mRNA expression in the paraventricular nucleus of a rat in the no-noise condition. Scale bar = 800 μ m. F. Representative autoradiograph of *c-fos* mRNA expression of the paraventricular nucleus of a rat in the loud noise stress condition.

Fig. 3.

Diagrammatic representations from the Paxinos and Watson (1998) atlas of brain sections with quantified regions of interest marked by thick geometric figures. Scale bars in each section = 5 mm. A. Pri: prelimbic cortex, IL: infralimbic cortex. B. LS: lateral septum. C. BNST: bed nucleus of the stria terminalis, MPA: medial preoptic area of the hypothalamus. D. PVN: paraventricular nucleus of the hypothalamus (each section individually traced), BLA: basolateral nucleus of the amygdala. E. Som: somatosensory cortex, Aud: auditory cortex, VP: ventral posterior nucleus of the thalamus. F. MGN: medial geniculate nucleus of the thalamus.

Fig. 4.

c-fos mRNA induction in stress-reactive subcortical brain structures. AM251 drug treatment significantly potentiated loud noise stress-induced neural activity in the anteroventral bed nucleus of the stria terminalis and medial preoptic area. Anteroventral bed nucleus of the stria terminalis: Significant main effects of loud noise stress (#, $p = 0.01$), AM251 drug treatment (*, $p < 0.01$), and significant interaction of noise stress and AM251 drug treatment (†, $p = 0.02$). Medial preoptic area: Significant main effects of loud noise stress (#, $p = 0.01$), AM251 drug treatment (*, $p < 0.01$), and significant interaction of noise stress and AM251 drug treatment (†, $p = 0.02$). Lateral septum: Significant main effects of loud noise stress (#, $p < 0.01$), AM251 treatment (*, $p = 0.05$), but no significant interaction between noise stress and AM251 treatment (†, $p = 0.10$). Basolateral amygdala: Significant main effects of loud noise stress (#, $p = 0.03$), AM251 drug treatment (*, $p < 0.01$), but no significant interaction of noise stress and AM251 drug treatment ($p = 0.41$).

Fig. 5. *c-fos* mRNA induction in thalamic regions. A contrasting pattern of interaction of loud noise stress and AM251 drug treatment was measured in both thalamic areas quantified (medial geniculate and ventral posterior nuclei) compared to other limbic areas. Systemic AM251 drug treatment resulted in inhibition of *c-fos* mRNA induction in both nuclei in response to loud noise stress. Medial geniculate nucleus: Significant effect of loud noise stress (#, $p = 0.02$). Ventral posterior nucleus: Significant interaction of loud noise stress and AM251 drug treatment (†, $p = 0.02$).

Fig. 6. *c-fos* mRNA induction patterns in the auditory, somatosensory, prelimbic and infralimbic cortices. AM251 drug treatment significantly increased *c-fos* mRNA induction in control rats in areas quantified. Significant effects of AM251 drug treatment: (*, Aud: $p < 0.01$, Som: $p = 0.01$, Prl: $p = 0.04$, IL: $p = 0.23$). Significant effects of loud noise stress: (#, Aud: $p < 0.01$, Prl: $p < 0.01$, IL: $p = 0.01$). This response was not further potentiated by AM251 drug treatment, but significant interactions between loud noise exposure and AM251 treatment were measured in the somatosensory cortex (†, $p = 0.04$), prelimbic cortex ($p = 0.05$) and infralimbic cortex ($p = 0.02$).